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Long-Pulse Dye Laser for Photodynamic Therapy: Investigations In Vitro and In Vivo

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Background and Objective: **Continuous wave lasers or incoherent lamps are used effectively for photodynamic therapy (PDT). As the mechanism of action of pulsed lasers in PDT is not known, we investigated the efficacy of PDT with 5-aminolevulinic acid (ALA) using a long-pulse (1.5 ms) tunable flashlamp-pumped pulsed dye laser (LPDL) in vitro and in vivo.**
Study Design/Materials and Methods: **HaCaT human keratinocytes were incubated with ALA (3 mmol/l) and irradiated (0–50 J/cm^2) using the LPDL at 585 nm, 595 nm, or 600 nm vs. an incoherent light source (580–740 nm). Topical ALA-PDT was performed on 24 patients with actinic keratoses (AK) on the head (n = 200) after incubation with a 20% ALA emulsion and irradiation by either an incoherent light source (160 mW/cm^2, 60–160 J/cm^2) or the LPDL (585 nm, 18 J/cm^2).**
Results: **Maximal cytotoxic effects in vitro were achieved using the LPDL at 585 nm or the incoherent lamp (50 J/cm^2). Sodium azide, a quencher of singlet oxygen, significantly reduced cell killing, suggesting that the cytotoxic effects are mainly mediated by singlet oxygen. This is supported by an increase of lipid peroxides as determined by malondialdehyde after adding D_2O. Complete remission was achieved in 79% of 100 AK treated by ALA and the LPDL and in 84% of 100 AK treated by ALA and the incoherent lamp. Pain during light treatment was significantly reduced by using the LPDL. Control lesions (LPDL without ALA) did not clear.**
Conclusion: **These results show the in vitro and in vivo efficacy of ALA-PDT using a pulsed light source mediated by singlet oxygen. Lasers Surg. Med. 25:51–59, 1999.** **© 1999 Wiley-Liss, Inc.**

Key words: actinic keratoses; 5-aminolevulinic acid; PDT; pulsed laser light; singlet oxygen; wavelength

INTRODUCTION

A photodynamic reaction is the excitation of a photosensitizer, mainly porphyrin derivatives, by visible light emitted by lamps or lasers. In clinical practice usually *continuous* wave light sources are used: either argon ion pumped dye lasers (λ 630 nm or 635 nm) or incoherent light sources (λ 540–700 nm) [1–3]. The excitation of the photosensitizer results in the generation of reactive oxygen species (ROS), particularly singlet oxygen. These ROS mediate cellular, e.g., lipid peroxidation, and vascular effects resulting in a direct or indirect cytotoxic effect on the treated cells [4]. However, the photodynamic efficacy of *pulsed* laser systems is currently not known in detail.

Since photodynamic therapy (PDT) using systemic photosensitizers like hematoporphyrin derivative leads to a long-lasting generalized skin photosensitivity, the topical application of photosensitizers, e.g., 5-aminolevulinic acid (ALA), is

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preferred for use in dermatology and therefore intensively studied [5]. In epithelial tumor cells ALA is metabolized to protoporphyrin IX (PpIX) [6]. Irradiation by red light selectively destroys skin tumors without harming the surrounding normal tissue. Topical photodynamic therapy with ALA has been shown to be effective for the treatment of actinic keratoses revealing complete remission rates of up to 90% after one single treatment [2,7–10].

The aim of this study was to investigate the in vitro efficacy of ALA-PDT using a long-pulse tunable flashlamp-pumped dye laser (LPDL) with a pulse duration of 1.5 ms and the mechanisms responsible for cytotoxicity. Moreover, the efficacy and tolerability of ALA-PDT using the pulsed laser (LPDL) for actinic keratoses was investigated as compared to an incoherent light source in vivo.

MATERIALS AND METHODS

Cell Lines and Culture Techniques

A human immortalized keratinocyte cell line (HaCaT) was maintained under standard conditions and plated in 96-well plates (7×10^4 cells per dish). After the removal of medium after 16 hours serum-free medium (100 μl) containing ALA (3 mmol/l, Merck AG, Darmstadt, Germany) was added, and cells were allowed to take up ALA for 24 hours. The medium containing ALA was removed and the cells were rinsed twice with phosphate-buffered saline (PBS) and then submerged in PBS. Irradiation was performed immediately afterwards.

Irradiation Procedure

Irradiation of the cell culture was performed using two different light sources: 1. A tunable long-pulse flashlamp-pumped pulsed dye laser (Sclerolaser®, Candela Corp., Wayland, Massachusetts, USA, pulse duration 1.5 ms), at different wavelengths ($\lambda = 585$ nm, $\lambda = 595$ nm, or $\lambda = 600$ nm), and different light doses (10, 20, 30, or 50 J/cm^2), 2. An incoherent light source [1] with a 1,200 watt metal halogen lamp (PDT 1200, Waldmann-Medizintechnik, Villingen-Schwenningen, Germany, emission wavelength λ_{em} 580–740 nm), with a light intensity of 40 mW/cm^2 and different light doses (10, 20, 30, or 50 J/cm^2).

For cell culture studies, 34 groups were formed ($n = 26$ for each group). The first served as a control and received neither sensitizer nor irradiation. The second group received ALA only. Groups 3–18 were irradiated only at the specific wavelengths, whereas groups 19–34 were sensitized with ALA (3 mmol/l) and irradiated using different light sources (LPDL at 585 nm, 595 nm, or 600 nm vs. incoherent light source) and different light doses (10, 20, 30, or 50 J/cm^2).

Cell Survival as Determined by the MTT Test

Following irradiation cells were maintained under normal culture conditions for another 24 hours. Viability of cells after ALA and/or light treatment was assessed by means of the 3-4,5-dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) assay [for details see 11]. Cells were washed once again with PBS and 25 μl of 5 mg/ml MTT dissolved in PBS was added. After removal of MTT after 2 hours, 100 μl of a 20% sodium dodecylsulfate solution was added for complete dissolution of the formazan dye. Optical density of wells was read with an enzyme-linked immunosorbent assay reader at 540 nm. The ratio of the optical densities of treated cells to untreated cells, which served as control, was referred to as cell viability (CV).

Lipid Peroxidation Assay

Unsaturated lipids are important targets of photooxidation of cellular membranes and malondialdehyde (MDA), a marker of lipid peroxidation, can be measured by the fluorometric thiobarbituric acid (TBA) assay [12]. After irradiation the cells were homogenized by ultrasound and centrifuged for 15 minutes. The suspension was mixed with phosphortungstic acid (10% in H_2O), sodiumdodecylsulfate (7% in H_2O), HCl (0.1 N), thiobarbituric acid (0.5% in H_2O), and shaken at a temperature of 95°C for 60 minutes. After cooling the MDA-TBA complex was extracted by n-butanol. The quantification of the MDA-TBA complex was performed by measuring its fluorescence at 553 nm with an MDA standard solution (0–3 nmol/ml) serving as control.

Effect of D_2O on Cell Killing

D_2O was added to the cells replacing H_2O 60 minutes prior to irradiation. HaCaT cells incubated with 3 mmol/l ALA for 24 hours were irradiated with the LPDL (585 nm, 50 J/cm^2) as described above but in presence of D_2O instead of H_2O. A control group received the same treatment but without irradiation. Cell viability was assessed as described earlier.

TABLE 1. Score for Classification of Actinic Keratoses

	(0) None	(1) Mild	(2) Moderate	(3) Severe	(4) Very severe
Infiltration	no infiltration	slight palpability	moderate infiltration	thick, easily to palpable	very thick
Keratosis	no keratosis	mild keratosis	moderate keratosis	thick keratosis	very thick keratosis

Effect of Sodium Azide on Cell Killing

Sodium azide, a potent physical quencher of reactive oxygen species [13–16], was added to the cell culture prior to irradiation. HaCaT cells incubated with 3 mmol/1 for 24 hours were irradiated by the LPDL (585 nm, 50 J/cm^2) as described earlier, but in presence of sodium azide (Merck, Darmstadt, Germany) at a concentration of 10, 50, or 100 mmol/l in PBS. The control group was treated in the same way but without irradiation. Cell viability was assessed as described above.

In Vivo Studies

Study design. The study was designed as a monocenter, open pilot study without control group. It was performed according to the German Drug Law and the European Guidelines on Good Clinical Practice (GCP) and approved by the local ethics committee. All patients gave their written informed consent prior to entry into the study.

Patient Selection

Male and female patients over 18 years of age, suffering from clinically diagnosed actinic keratoses (AK), were considered for entry into the study. AK were selected with regard to the following inclusion criteria: the number of 3–20 lesions localised on the scalp and/or face. AK on the trunk or extremities were excluded due to their poor response to topical PDT with ALA [10,17].

Treatment Protocol

The baseline examination included the evaluation of anamnestic and demographic data as well as the inclusion criteria and the selection of the test lesions. Lesions were judged by an established semiquantitative score regarding keratosis and infiltration (Table 1) [17].

For treatment, AK were covered with a W/O-emulsion containing 20% aminolevulinic acid-HCl (Medac, Hamburg, Germany) as the active ingredient and kept under occlusion impervious to light. After 6 hours, therapeutic irradiation was performed. In each patient about 50% of the lesions were irradiated by an incoherent light source (PDT 1200) emitting red light among 580–740 nm with a light intensity of 160 mW/cm^2 and a total light dose of 60–160 J/cm^2. The remaining lesions were irradiated by the LPDL at a wavelength of 585 nm (18 J/cm^2, pulse duration 1.5 ms, pulse diameter 5 mm). When using the LPDL, each AK smaller than 5 mm in diameter was treated with a single pulse, whereas several pulses (n = 2–4) were needed to cover the whole lesion in larger AK. One additional control lesion was treated by the LPDL at 585 nm (18 J/cm^2) in each patient without prior incubation with ALA. Pain during irradiation with the laser and the lamp was judged immediately after irradiation using a visual analog pain scale ranging from 0 (no pain) to 10 (maximal pain). Using the lamp, light doses ranged from 60 to 160 J/cm^2, since irradiation was stopped when the stinging or burning sensation became intolerable for the patient (pain score 10).

The therapeutic efficacy was monitored and documented 4 days, 28 days, and every month after treatment. Efficacy was judged using a semiquantitative score (Table 1) for keratosis and infiltration of AK before and 28 days after treatment.

Side effects of therapy such as erythema, edema, infection, or other local side effects were documented, as well as the final cosmetic results.

Statistics

The StatView data analysis system (Abacus Concepts Inc., Berkeley, CA) was used for statistical analysis of in vitro data. Results are expressed as mean and standard deviation. Parametric tests (Scheffe's-test) were used for the in vitro data and nonparametric tests were used for the in vivo data. For description, *P*-values (two-tailed Student's t-test) were given. Probability values smaller than 5% were considered as significant.

RESULTS

Cell Culture Studies

Irradiation alone (10–50 J/cm^2) did not affect cell viability. Cells treated with ALA and the in-

coherent light source showed a significant reduction of cell viability (CV) as compared to the nonirradiated control. Reduction of CV was significantly higher when irradiating with 20, 30, or 50 J/cm^2 as compared to 10 J/cm^2. There was no significant difference between the light doses 20, 30, or 50 J/cm^2, CV ranged between 12.1–10.8%. Using the LPDL at 585 nm, a significant reduction of CV was achieved for all light doses compared to the nonirradiated control. The difference between 10 and 20 J/cm^2 was not significant, 30 J/cm^2 was significantly more effective in cell killing (CV 31.3%) as compared to 20 J/cm^2 (CV 54.4%) or 10 J/cm^2 (CV 56.6%). The difference between 30 J/cm^2 and 50 J/cm^2 (CV 17.2%) was also substantial. Using the LPDL at 595 nm or 600 nm resulted in a similar light dose dependent effect on cell viability. As compared to the nonirradiated control, all light doses led to a significant reduction of CV.

Maximal cytotoxic effects were achieved using the LPDL at 585 nm at a light dose of 50 J/cm^2 or the incoherent light source at light doses between 20–50 J/cm^2. There was no significant difference between the incoherent lamp (CV 17.2%) and the laser at 585 nm (CV 10.8%) at a light dose of 50 J/cm^2, whereas the LPDL at 595 nm (CV 28.2%) and at 600 nm (CV 46.8%) yielded a significantly lower cytotoxicity as compared to the lamp (Fig. 1).

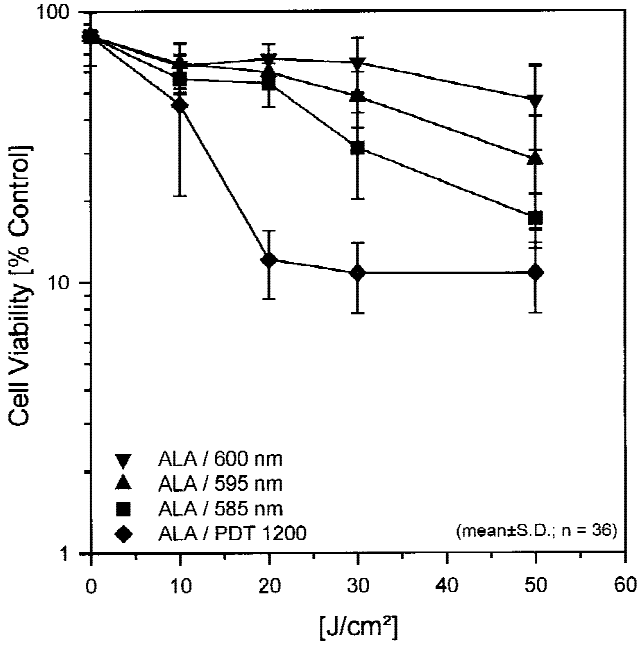

Fig. 1. Cell viability of HaCaT keratinocytes after incubation with 3 mmol/l ALA and irradiation using an incoherent light source (PDT 1,200) vs. the LPDL at 585 nm, 595 nm, and 600 nm at different light doses (10, 20, 30, and 50 J/cm^2). Maximal cytotoxic effects were achieved using the LPDL at 585 nm or the incoherent light source with a light dose of 50 J/cm^2. The LPDL at 595 nm or 600 nm yielded a significantly lower cytotoxicity.

Lipid Peroxidation Assay

The amount of MDA (Fig. 2) increased from 1.17 ± 0.4 nmol/10^6 cells to 20.47 ± 7.8 nmol/10^6 cells after light irradiation (LPDL 585 nm, 50 J/cm^2) of HaCaT cells incubated with 3 mmol/1 ALA for 24 hours. Using D_2O lipid peroxides accumulated dramatically from 1.12 ± 0.0 nmol/10^6 cells to 75.61 ± 2.1 nmol/10^6 cells. The increase of lipid peroxides after addition of D_2O to the cell culture suggests that reactive oxygen species mediate the cytotoxic effects.

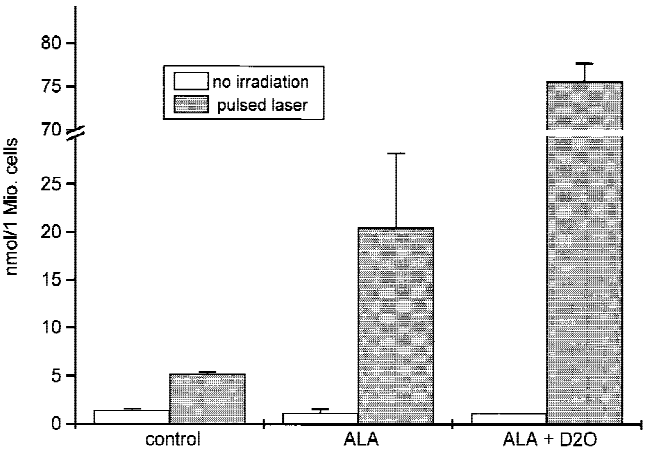

Fig. 2. Lipid peroxidation assay: addition of malondialdehyde (MDA) after incubation of HaCaT keratinocytes with 3 mmol/l ALA, irradiation with the LPDL at 585 nm and 50 J/cm^2. The increase of lipid peroxides after addition of D_2O as measured by means of the fluorometric thiobarbituric acid assay suggests that cell killing is mediated by singlet oxygen.

Effect of Sodium Azide on Cell Killing

Figure 3 shows the effect of incubation of HaCaT cells with ALA (3 mmol/l) and sodium azide (10, 50, 100 mmol/l), a potent singlet oxygen quencher, using the LPDL at 585 nm (50 J/cm^2) for irradiation. Using ALA and laser irradiation only, cell viability (CV) was 26.9 ± 8.7% of the untreated control (CV 100%). The cell killing of ALA after irradiation was substantially reduced in the presence of sodium azide as shown by a significant increase in cell viability (Fig. 3). No

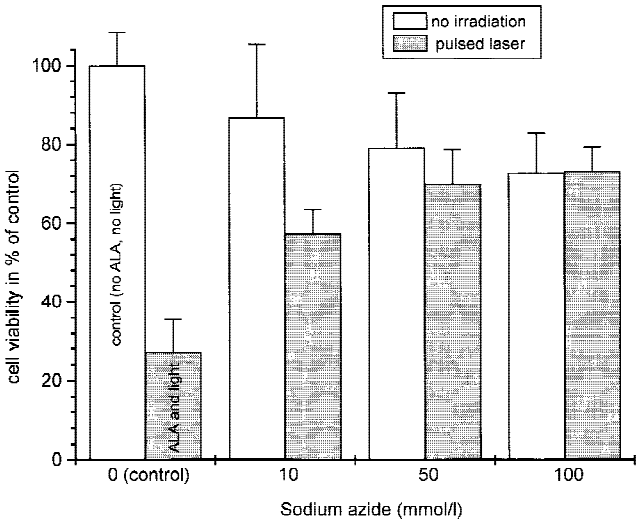

Fig. 3. Effects of the singlet oxygen quenchers sodium azide on cell killing after incubation of HaCaT cells with ALA (3 mmol/l) and addition of sodium azide (10, 50, 100 mmol/l) using the LPDL at 585 nm (50 J/cm^2) for irradiation. Reduction of cell killing by addition of sodium azide indicates that singlet oxygen is the mediator for cytotoxicity.

significant difference was shown, if sodium azide was added 0, 45, or 180 minutes prior to irradiation.

In Vivo Studies

A total of 200 AK in 24 patients (4 female and 20 male patients, mean age 72 ± 7 years) were treated. 100 AK were irradiated by the incoherent lamp, whereas 100 AK were irradiated by the LPDL. All AK were localized on the head or face.

The initial mean score of the AK treated by the incoherent lamp was 4.35 ± 0.8 and 4.30 ± 0.8 of the AK treated by the LPDL. Twenty eight days after PDT the mean score was significantly ($P <$ 0.0001) lower for both light sources (lamp: 0.27 ± 0.7, laser: 0.33 ± 0.7). Of the lesions, 84% treated by the incoherent lamp and 79% treated by the LPDL showed complete remission 28 days after topical PDT (Table 2).

The only adverse side effect of this therapy was a stinging and burning pain during and after irradiation. There was a highly significant difference ($P < 0.001$) between the pain during treatment using the incoherent lamp (mean score 7.7 ± 2.3) and using the LPDL (mean score 3.2 ± 1.0). In 9 patients, irradiation by the incoherent lamp was stopped before reaching a light dose of 160 J/cm^2 due to severe discomfort (pain score 10, light dose 60–160 J/cm^2).

All AK treated with ALA and irradiated by laser or lamp showed erythema and crusting 2–4 days after irradiation, which lasted for about 10–14 days (Fig. 4A–C). Immediately after irradiation by the LPDL, a haemorrhagic discoloration occurred in the treated area also completely resolving within 10–14 days.

All patients showed excellent cosmetic results, independent of the light source used. Only slight erythema was noted in some patients 28 days after PDT, which resolved within a few months thereafter.

DISCUSSION

Although both mechanisms of action in photodynamic therapy, photooxidation type I and type II, may take place at the same time, singlet oxygen is supposed to play a major role when using the photosensitizer Photofrin in combination with a *continuous* light source [18]. In contrast to continuous light sources, the photodynamic efficacy of *pulsed* laser systems is still not understood in detail. Pulsed light sources (e.g., flashlamp pumped dye lasers) may induce photon saturation effects of photosensitizers resulting in suboptimal photosensitization [19]. A saturation phenomenon could also explain the ineffective in vitro photosensitization of the aluminum sulfonated phthalocyanine when using a pulsed copper vapor pumped dye laser with a short pulse width of 9 ns [20]. However, a pulsed copper vapor pumped dye laser with a long pulse width of 2 μs was comparable to a continuous wave argon ion pumped dye laser in an animal model using a phthalocyanine as photosensitizer [21]. Sterenborg and van Gemert [22] made a theoretical analysis of PDT with pulsed light sources using a mathematical model describing the time-dependent excitation and deexcitation of a photosensitizer molecule using haematoporphyrin. They calculated that the effectiveness of pulsed excitation in PDT is identical to that of continuous wave for peak fluence rates below 4 × 10^8 W/cm^2. Above this threshold the effectiveness should drop significantly. The peak fluence rate used in our experiments was below the calculated threshold (e.g., approx. 3 × 10^4 W/cm^2). This hypothesis is further supported by experimental data. Thus, several authors compared pulsed and continuous wave lasers for systemic PDT using porphyrins in an animal model showing that both light sources were indifferent regarding the PDT effect or cure rate of tumors

TABLE 2. Clinical Outcome After ALA-PDT of Actinic Keratoses: No Difference Between Both Light Sources Regarding Complete Remission of Actinic Keratoses, but Significant Pain Reduction When Using the LPDL

Light source	Number of AK [n]	CR [%]	Score (infiltration + keratosis) of AK before PDT [mean ± SD]	Score (infiltration + keratosis) of AK after PDT [mean ± SD]	Pain score [mean ± SD]
Incoherent light source	100	84	4.35 ± 0.8	0.27 ± 0.7	7.7 ± 2.3
LPDL	100	79	4.30 ± 0.8	0.33 ± 0.7	3.2 ± 1.0

such as papillomas, endobronchial tumors, or squamous cell carcinomas [23–26].

The first aim was to determine whether singlet oxygen is responsible for cell killing in photodynamic therapy when using a pulsed light source in vitro. Cell killing was significantly reduced by the addition of sodium azide to the cells suggesting that cell killing of HaCaT cells by ALA and a pulsed light source is mainly mediated by singlet oxygen. Thus, photothermal effects are not the major cause for cell killing by ALA and a LPDL, because otherwise quenchers of singlet oxygen like sodium azide should fail to reduce ALA- and laser-induced phototoxicity. Moreover, the increase in lipid peroxides in vitro as measured by means of the fluorometric thiobarbituric acid assay after adding D_2O to the cell culture medium [12] supports this finding (Fig. 2). Thus, ALA and irradiation with a long-pulse LPDL induce the formation of reactive oxygen species in vitro.

Maximal cytotoxic effects of ALA-PDT in cell culture were achieved using the LPDL at 585 nm or the incoherent light source at a light dose of 50 J/cm^2, whereas the LPDL at 595 nm and 600 nm yielded a significantly lower cytotoxicity. This wavelength dependent effect can be explained by the absorption spectrum of protoporphyrin IX (PpIX) (Fig. 5), which meets an absorption maximum near 585 nm, whereas at 595 nm and 600 nm the absorption of PpIX is markedly lower.

Based on the in vitro results we proved the efficacy of ALA-PDT using the LPDL in vivo on 24 patients with actinic keratoses on the head (n = 200). Twenty-eight days after PDT, complete remission was achieved in 79% of 100 AK treated with the LPDL and in 84% of 100 AK treated with the incoherent lamp. However, pain during light treatment could be markedly reduced when using the LPDL because the short exposure time (spot size 5 mm, single pulse duration 1.5 ms) caused significantly less discomfort as compared to a 6–16 minute irradiation using the incoherent lamp (single irradiation area: 10 × 10 cm). In contrast to most other treatment modalities for AK, a single treatment with PDT is effective in clearing up about 80% of the lesions with an excellent cosmetic result. Clinically, no difference was found regarding the effect of both light sources. Due to the specific absorption characteristics of the LPDL for intravasal oxyhemoglobin, an additional vascular effect should be considered probably inducing remission of the lesions due to disruption of the supplying tumor microvasculature. However, control lesions treated with the LPDL only did not respond to laser therapy suggesting that the PDT effect in vivo is mainly mediated by generation of reactive oxygen species but not by vascular effects of the laser.

Despite the benefit of significant pain reduction, the LPDL has various disadvantages such as: high costs, a need for special electrical supplies, no portability, and frequent optical alignment and dye replacement. Furthermore, long-term follow-up after ALA-PDT using the LPDL in comparison with the incoherent lamp has not yet been investigated. Thus, irradiation using the lamp allows one to hit subclinical lesions by treating the apparent actinic keratoses and the perilesional sun-damaged skin on head and neck with ALA-PDT. When using the LPDL with a pulse diameter of just 5 mm, only clinically evident lesions can be treated with single pulses.

For a flashlamp pumped dye laser (585 nm, 450 μs, 6–8 J/cm^2), an overall vessel-wall coagulation depth to a maximum of 0.65 mm (mean 0.37 mm) was shown [27]. Tissue penetration of laser light of 585 nm is not as deep as at 630 or 635 nm—the wavelengths most often used for laser irradiation in PDT [2]. However, this fact is not of relevance for the treatment of actinic keratoses, which are very superficial lesions (<0.37 mm) located within the epidermis of the skin. In case of basal cell carcinoma or other invasive tumors, the lower penetration depth of light at 585 nm could

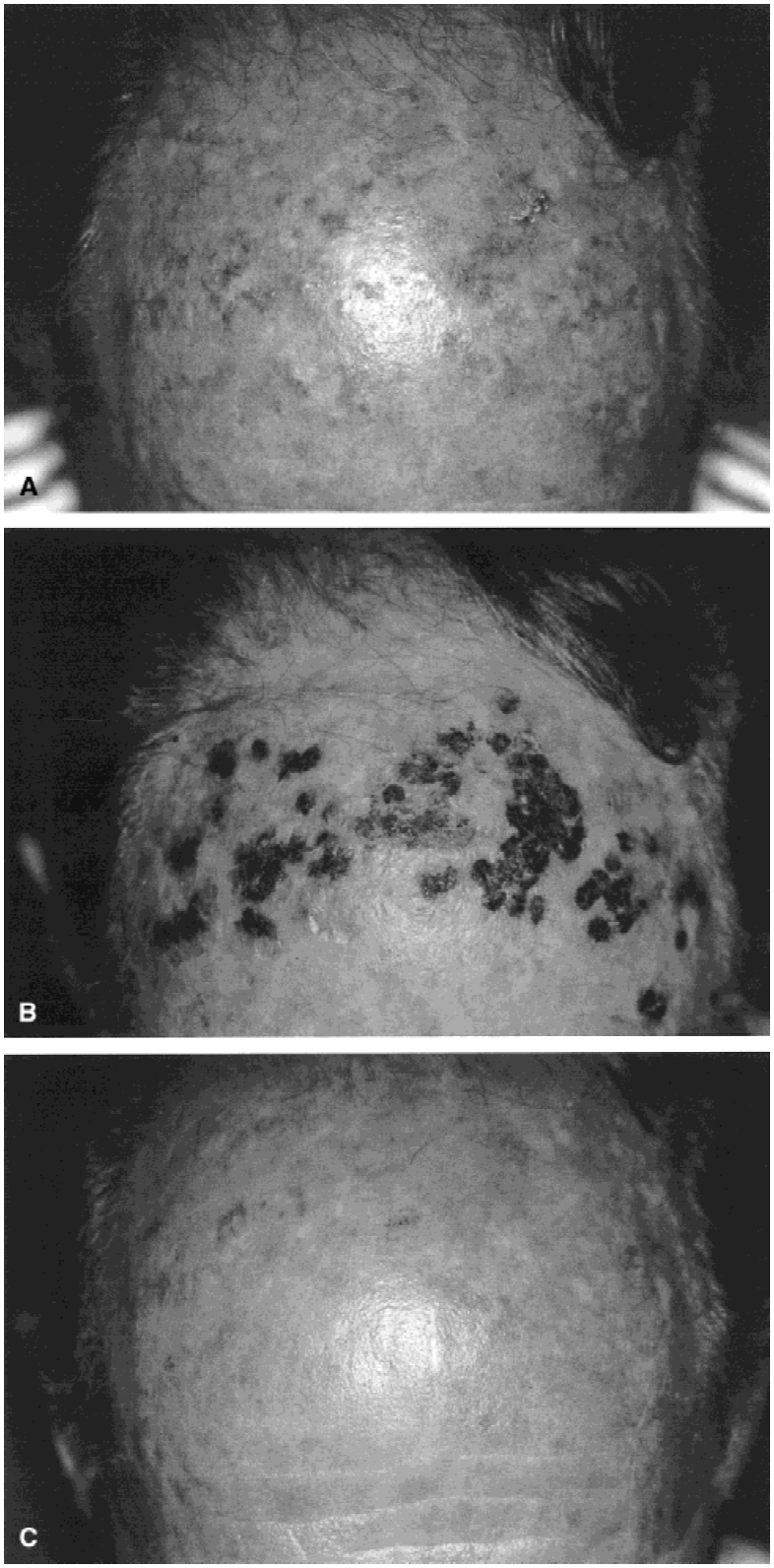

Fig. 4. Sixty-five-year-old male patient with multiple actinic keratoses on the forehead. **4A:** Before treatment. **4B:** Six days after PDT using the incoherent lamp on the right side and the LPDL on the left side of the forehead. **4C:** Three months after PDT; complete remission of the treated lesions.

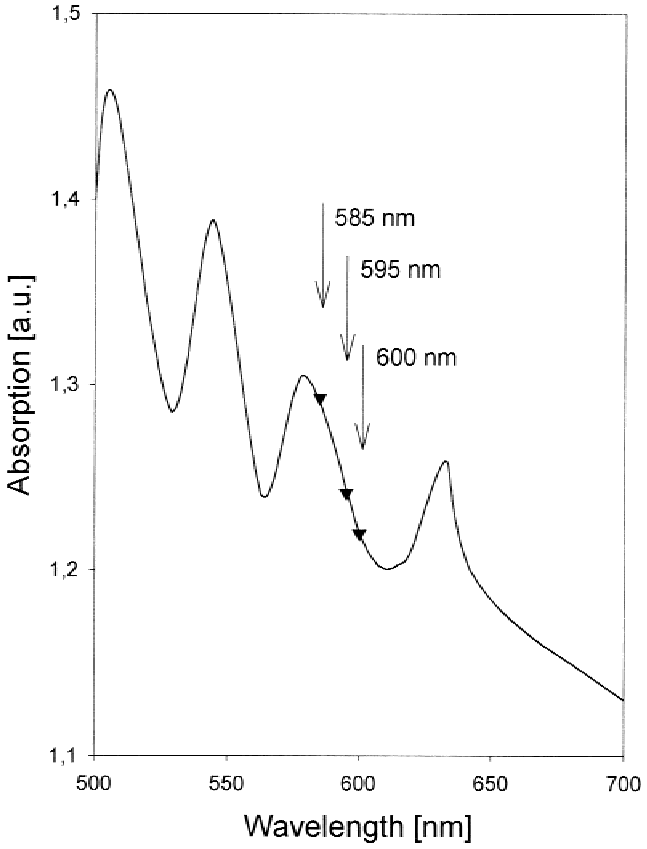

Fig. 5. Absorption bands of protoporphyrin IX in cell culture medium, markedly higher absorption of PpIX at a wavelength of 585 nm as compared to 595 nm and 600 nm.

be a limiting factor regarding a complete remission of such lesions after PDT.

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